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Date: May 9, 2002

Eileen M. Ebel

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

TECH CENTER 1600/2900

In re application of:

Group: 1644

Michael Brandt, et al.

Serial No.: 10/081,309

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Filed: February 21, 2002

For: PEG CONJUGATES OF NK4

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May 9, 2002

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Attached please find the certified copy of the foreign application from which priority is claimed for this case:

<u>Country</u>	<u>Application No.</u>	<u>Filing Date</u>
Europe	01104640.6	February 23, 2001

Respectfully submitted,

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Reg. No. 37,316

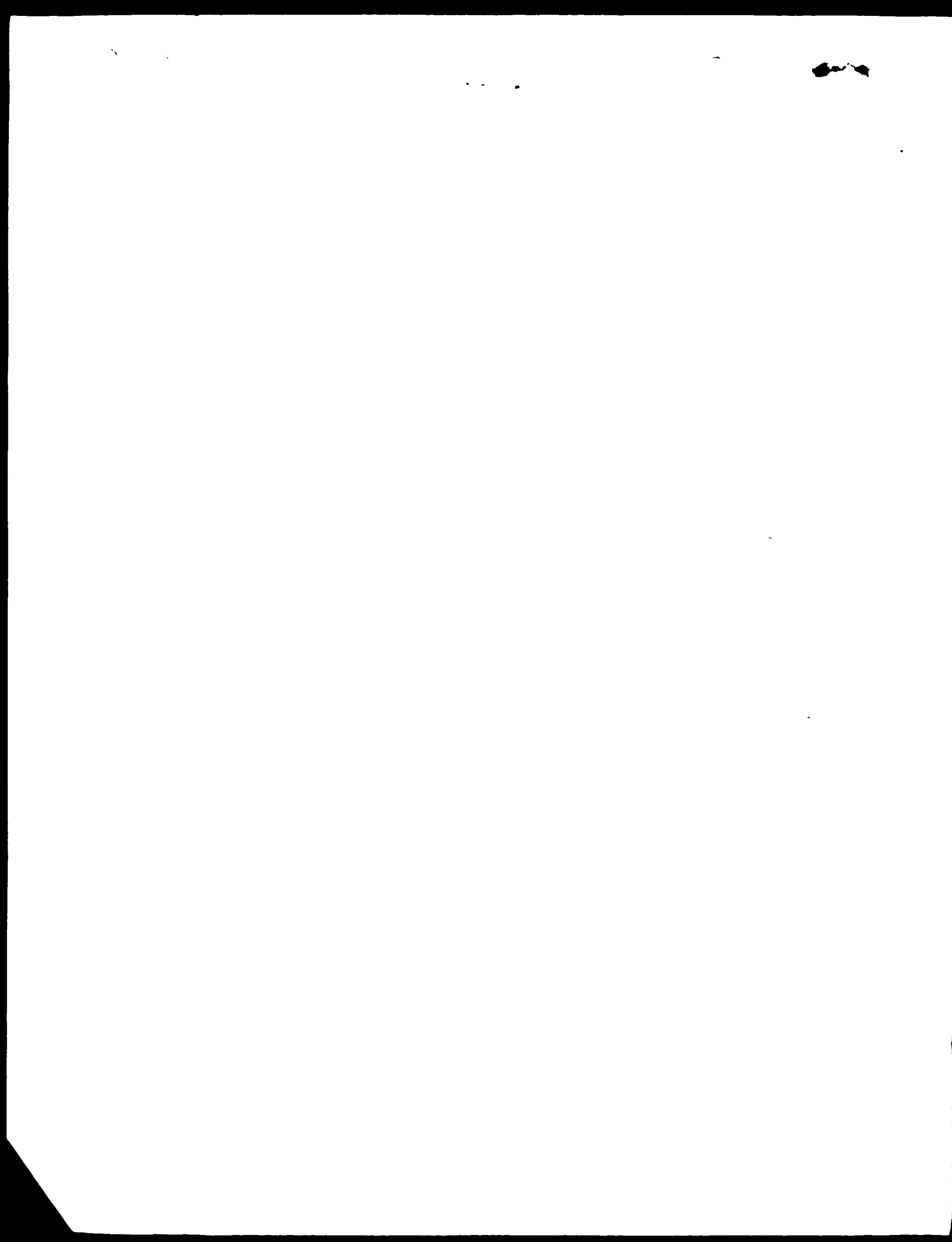
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Patentanmeldung Nr. Patent application No. Demande de brevet n°

01104640.6

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

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Sheet 2 of the certificate
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Application no:
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Titre de l'invention:
PEG-conjugates of HGF-NK4

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F. HOFFMANN-LA ROCHE AG

Case 20859 EP

PEG-conjugates of HGF-NK4

This invention relates to conjugates of the N-terminal four kringle-containing fragment of hepatocyte growth factor (NK4) with polyethylene glycol (PEG), pharmaceutical compositions thereof, methods for the production and methods for use.

Background of the Invention

- 5 Hepatocyte growth factor (HGF/SF) is a polypeptide identified and purified by Nakamura, T., et al., Biochem. Biophys. Res. Commun. 22 (1984) 1450-1459. It was further found that hepatocyte growth factor is identical to scatter factor (SF), Weidner, K.M., et al., Proc. Natl. Acad. Sci. USA 88 (1991) 7001-7005. HGF is a glycoprotein involved in the development of a number of cellular phenotypes including proliferation, mitogenesis,
10 formation of branching tubules and, in the case of tumor cells, invasion and metastasis. For a status review, see Stuart, K.A., et al., International Journal of Experimental Pathology 81 (2000) 17-30.

- Both rat HGF and human HGF have been sequenced and cloned (Miyazawa, K. et al., Biochem. Biophys. Res. Comm. 163 (1989) 967-973; Nakamura, T., et al., Nature 342
15 (1989) 440-443; Seki, T., et al., Biochem. and Biophys. Res. Comm. 172 (1990) 321-327; Tashiro, K., et al., Proc. Natl. Acad. Sci. USA 87 (1990) 3200-3204; Okajima, A., et al., Eur. J. Biochem. 193 (1990) 375-381).

- U.S. Patent No. 5,977,310 describes PEG-modified HGF. Such PEG-modified HGF has a prolonged clearance in vivo and has the same physiological activity as HGF. However,
20 according to U.S. Patent No. 5,977,310, it is only possible to prolong the half life of HGF from 59.2 minutes to 76.7 minutes or 95.6 minutes, respectively (see Example 5 of U.S. Patent No. 5,977,310). It is further suggested in this patent that the molar amount of the PEG reagent may be selected from the range of from 5 to 100 times of the molar weight of HGF. In the case of modifying an amino group of lysine or the N-terminus of protein, a
25 preferred molar range of the PEG reagent is of from 10 to 25 times of the molar weight of HGF. The molecular weight of the attached PEG chain was about 10 kDa.

- It was further found that an HGF/SF fragment, termed NK4, consisting of the N-terminal hairpin domain and the four kringle domains of HGF/SF has pharmacological properties that are completely different from those of HGF/SF, and is an antagonist to the influence of
30 HGF/SF on the motility and the invasion of colon cancer cells, and is, in addition, an

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angiogenesis inhibitor that suppresses tumor growth and metastasis (Parr, C., et al., Int. J. Cancer 85 (2000) 563-570; Kuba, K., et al., Cancer Res. 60 (2000) 6737-6743; Date, K., et al., FEBS Letters 420 (1997) 1-6; Date, K., et al., Oncogene 17 (1989) 3045-3054).

5 According to the state of the art, NK4 is, however, not a substance that appears suitable for easy therapeutic use in humans. As emerges from Kuba, K., et al., Cancer Res. 60 (2000) 6737-6743, in animal experiments, for detecting an effect of NK4 on lung metastases, NK4 had to be infused continuously over a period of two weeks.

10 It is known that the attachment of polymers to certain polypeptides may increase the serum half life of such polypeptides. This was found, for example, for pegylated Interleukin-6 (EP 0 442 724) or Interleukin-2 (WO 90/07938) and erythropoietin (WO 01/02017). However, the attachment of polyethylene glycol and other polymers did not necessarily lead to prolongation of their serum half lives. It is known, for example, that the conjugation of different polyethylene glycols to Interleukin-8, G-CSF and other interleukins results in the production of molecules with impaired properties (Gaertner, H.F., and Offord, R.E.,
15 Bioconjugate Chem. 7 (1996) 38-44; Mehvar, R., J. Pharm. Pharm. Sci. 3 (1) (2000) 125-136). Thus, the outcome of a pegylation of a polypeptide is highly unpredictable.

It was an object of the present invention to find an improved pharmaceutical composition for NK4, which composition can be administered as only a few, bolus applications per week and which is capable of suppressing tumor growth, angiogenesis and metastasis.

20 Summary of the Invention

The present invention provides NK4 conjugates consisting of NK4 being covalently linked to from one to three polyethylene glycol (PEG) groups (pegylated NK4).

It has been found, surprisingly, that pegylated, preferably monopegylated, NK4 according to the invention has superior properties in regard to the therapeutic applicability.

25 The invention further comprises a method for the production of pegylated NK4.

The invention further comprises pharmaceutical compositions containing pegylated NK4.

The invention further comprises methods for the production of pharmaceutical compositions containing pegylated NK4.

- 4 -

or mixtures thereof.

"Pegylated NK4" as used herein therefore means that NK4 has attached covalently one, two or three polyethylene glycol groups. The groups can be attached at different sites of the NK4 molecule, preferably, however, at the most reactive sites, e.g., the lysine side chains. Due to the synthesis method used, "pegylated NK4" can consist of a mixture of mono-, di- and/or tripegylated NK4, whereby the sites of pegylations can be different in different molecules or can be substantially homogeneous in regard to the amount of polyethylene glycol side chains per molecule and/or the site of pegylation in the molecule. Isolation and purification of such homogeneous preparations of pegylated NK4 can be performed by usual purification methods, preferably size exclusion chromatography.

"Substantially homogeneous" as used herein means that the only PEG-NK4 conjugate molecules produced, contained or used are those having one, two or three PEG group(s) attached and/or are homogeneous in regard to the site of pegylation. The preparation may contain unreacted (i.e., lacking PEG group) protein. As ascertained by peptide mapping and N-terminal sequencing, one example below provides for the preparation which is at least 90% PEG-NK4 conjugate (preferably monopegylated) and at most 2 % unreacted protein.

"Monopegylated" as used herein means that NK4 is pegylated at only one group per NK4 molecule, whereby only one PEG group is attached covalently at this site and the sites of attachment can vary within the monopegylated species.

The monopegylated NK4 is at least 90% of the preparation, and most preferably, the monopegylated NK4 is 92%, or more, of the preparation. The monopegylated NK4 preparations according to the invention are therefore homogeneous enough to display the advantages of a homogeneous preparation, e.g., in a pharmaceutical application.

In a further preferred embodiment of the invention, there is provided a mixture of pegylated NK4 conjugates (especially preferred are monopegylated conjugates), wherein monopegylation has occurred at different sites (at different amino acids) of the NK4 molecules.

30 The PEG polymer molecules used according to the invention have a molecular weight of about 10 to 40 kDA (by "molecular weight" as used here there is to be understood the mean

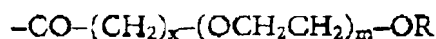
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molecular weight of the PEG; the term "about" indicates that in said PEG preparations, some molecules will weigh more and some less than the stated molecular weight).

According to the invention, preferably a method is provided for the production of a substantially homogeneous monopegylated NK4.

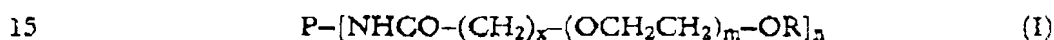
5 Pegylation of NK4 can be performed according to the methods of the state of the art.

In a preferred embodiment of the invention, said NK4 is covalently linked to "n" poly(ethylene glycol) groups of the formula



with the $-\text{CO}$ (i.e. carbonyl) of each poly(ethylene glycol) group forming an amide bond
10 with one of the amino groups of NK4; R being lower alkyl; x being 2 or 3; m being from about 200 to about 950; n being from 1 to 3; and n and m being chosen together so that the molecular weight of the conjugate minus the NK4 protein is from 10 kDa to 40 kDa, preferably from 20 to 40 kDa.

More specifically, the above conjugates may be represented by formula (I)



wherein P is the group of an NK4 protein as described herein, (i.e. without the amino group or amino groups which form an amide linkage with the carbonyl shown in formula (I); and wherein R is lower alkyl; x is 2 or 3; m is from about 200 to about 950; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the NK4
20 protein is from 10 kDa to 40 kDa, preferably from 20 to 40 kDa. As used herein, the given ranges of "m" merely have an orientational meaning. The ranges of "m" are determined in any case, and exactly, by the molecular weight of the PEG group(s).

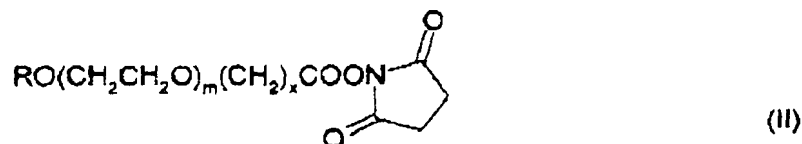
As used herein, "lower alkyl" means a linear or branched alkyl group having from one to six carbon atoms. Examples of lower alkyl groups include methyl, ethyl and isopropyl. In
25 accordance with this invention, R is any lower alkyl. Conjugates in which R is methyl are preferred.

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The symbol "m" represents the number of ethylene oxide groups (OCH₂CH₂) in the poly(ethylene oxide) group. A single PEG subunit of ethylene oxide has a molecular weight of about 44 daltons. Thus, the molecular weight of the conjugate (excluding the molecular weight of the NK4) depends on the number "m". In the conjugates of this invention "m" is from about 200 to about 950 (corresponding to a molecular weight of about 10 kDa to about 40 kDa), preferably from about 450 to about 950 (corresponding to a molecular weight of about 20 kDa to about 40 kDa). The number m is selected such that the resulting conjugate of this invention has a physiological activity comparable to unmodified NK4, which activity may represent the same as, more than, or a fraction of the corresponding activity of unmodified NK4. A molecular weight of "about" a certain number means that it is within a reasonable range of that number as determined by conventional analytical techniques. The number "m" is selected so that the molecular weight of each poly(ethylene glycol) group covalently linked to the NK4 protein is from about 10 kDa to about 40 kDa, and is preferably about 20 kDa to about 40 kDa, however the maximum molecular weight of all poly(ethylene glycol) groups together not exceeding 40 kDa.

In the conjugates of this invention, the number "n" is the number of polyethylene glycol groups covalently bound to free amino groups (including ε-amino groups of a lysine amino acid and/or the amino-terminal amino group) of an NK4 protein via amide linkage(s). A conjugate of this invention may have one, two, or three PEG groups per molecule of NK4. "n" is an integer ranging from 1 to 3, preferably "n" is 1 or 2, and more preferably "n" is 1.

The compound of formula (I) can be prepared, for example, from a known activated polymeric material:



in which R and m are as described above, by condensing the compound of Formula II with the NK4 protein. Compounds of formula (II) in which x is 3 are alpha-lower alkoxybutyric acid succinimidyl esters of poly(ethylene glycol) (lower alkoxy-PEG-SBA). Compounds of formula (II) in which x is 2 are alpha-lower alkoxypropionic acid succinimidyl esters of poly(ethylene glycol) (lower alkoxy-PEG-SPA). Any conventional method of reacting an activated ester with an amine to form an amide can be utilized. In

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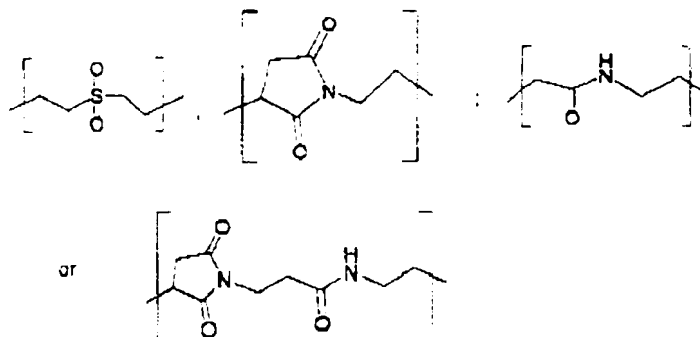
the reaction described above, the exemplified succinimidyl ester is a leaving group causing the amide formation. The use of succinimidyl esters such as the compounds of formula II to produce conjugates with proteins are disclosed in U.S. Patent No. 5,672,662, issued September 30, 1997 (Harris, et al.).

- 5 Human NK4 contains 30 free ε-amino groups of 30 lysine residues. When the pegylation reagent was combined with a SBA compound of Formula II, it has been found that at a pH of about 7.0, a protein:PEG ratio of about 1:3, and a reaction temperature of from 20-25 °C, a mixture of mono-, di-, and trace amounts of the tri-pegylated species were produced. When the protein:PEG ratio was about 1:1, primarily the mono-pegylated species is
10 produced. By manipulating the reaction conditions (e.g., ratio of reagents, pH, temperature, protein concentration, time of reaction etc.), the relative amounts of the different pegylated species can be varied.

- Monopegylated NK4 can also be produced according to the methods described in WO 94/01451. WO 94/01451 describes a method for preparing a recombinant polypeptide
15 with a modified terminal amino acid alpha-carbon reactive group. The steps of the method involve forming the recombinant polypeptide and protecting it with one or more biologically added protecting groups at the N-terminal alpha-amine and C-terminal alpha-carboxyl. The polypeptide can then be reacted with chemical protecting agents to selectively protect reactive side chain groups and thereby prevent side chain groups from being
20 modified. The polypeptide is then cleaved with a cleavage reagent specific for the biological protecting group to form an unprotected terminal amino acid alpha-carbon reactive group. The unprotected terminal amino acid alpha-carbon reactive group is modified with a chemical modifying agent. The side chain protected terminally modified single copy polypeptide is then deprotected at the side chain groups to form a terminally modified
25 recombinant single copy polypeptide. The number and sequence of steps in the method can be varied to achieve selective modification at the N- and/or C-terminal amino acid of the polypeptide.

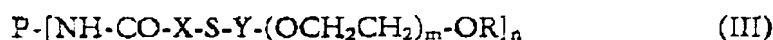
- Further preferred conjugates according to the invention consist of NK4 protein being covalently linked to from one to three lower-alkoxy poly(ethylene glycol) groups, each
30 poly(ethylene glycol) group being covalently linked to the protein via a linker of the formula $-C(O)-X-S-Y-$ with the C(O) of the linker forming an amide bond with one of said amino groups, X is $-(CH_2)_k-$ or $-CH_2(O-CH_2-CH_2)_k-$, k is from 1 to 10, Y is

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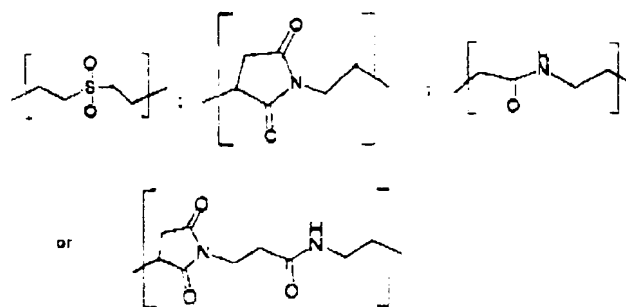
the average molecular weight of each poly(ethylene glycol) moiety is from about 10 kDa to about 40 kDa, not exceeding 40 kDa for all poly(ethylene glycol) moieties, and the molecular weight of the conjugate is from about 62 kDa to about 92 kDa at a molecular weight of 52 kDa for NK4 polypeptide, or from about 67 kDa to about 97 kDa at a molecular weight of 57 kDa for NK4 glycoprotein.

This NK4 species may also be represented by formula (III)



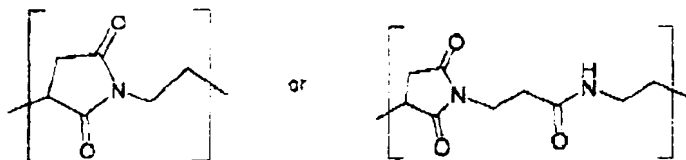
wherein R may be any lower alkyl, by which is meant a linear or branched alkyl group having from one to six carbon atoms such as methyl, ethyl, isopropyl, etc. A preferred alkyl is methyl. X may be $-(CH_2)_k-$ or $-CH_2(O-CH_2-CH_2)_k-$, wherein k is from 1 to about 10. Preferably, k is from 1 to about 4, more preferably, k is 1 or 2. Most preferably, X is $-(CH_2)$.

In formula III, Y is

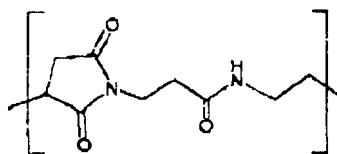


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preferably



5 more preferably

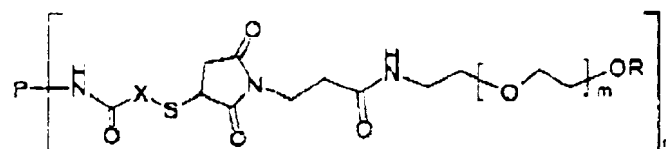
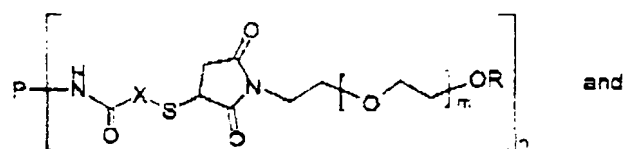


In formula (III), the number m is selected such that the resulting conjugate of formula (III) has a physiological activity comparable to unmodified NK4, which activity may represent the same as, more than, or a fraction of the corresponding activity of unmodified NK4. m represents the number of ethylene oxide chains in the PEG unit. A single PEG subunit of $-(OCH_2CH_2)-$ has a molecular weight of about 44 daltons. Thus, the molecular weight of the conjugate (excluding the molecular weight of the NK4) depends on the number m . A molecular weight of "about" a certain number means that it is within a reasonable range of that number as determined by conventional analytical techniques. m is therefore an integer ranging from about 200 to about 950 (corresponding to a molecular weight of from about 10 to 40 kDa), preferably m is from about 450 to about 950 (about 20 to 40 kDa).

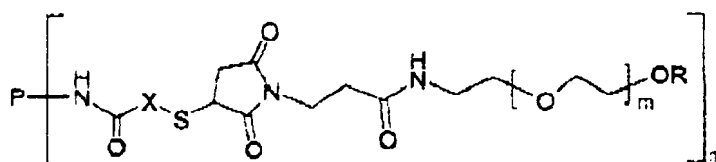
In formula (III), the number n is the number of ϵ -amino groups of a lysine amino acid in a NK4 protein covalently bound to a PEG unit via an amide linkage. A conjugate of this invention may have one, two, or three PEG units per molecule of NK4. n is an integer ranging from 1 to 3, preferably n is 1 or 2, and more preferably n is 1.

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Preferred NK4 proteins of formula (III) are represented by the formulae:



Most preferred NK4 protein products are represented by the formula:



5 These NK4 proteins may be prepared by

(a) covalently reacting an ε-amino group of a lysine amino acid of an NK4 protein represented by the formula, P-[NH₂]_n, with a bi-functional reagent represented by the formula, Z-CO-X-S-Q, to form an intermediate with an amide linkage represented by the formula:

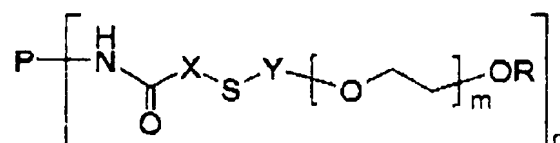
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wherein P is an NK4 protein less the amino group which forms an amide linkage; n is an integer ranging from 1 to 3; Z is a reactive group, e.g. a carboxylic-NHS ester; X is -(CH₂)_k- or -CH₂(O-CH₂-CH₂)_k-, wherein k is from 1 to about 10; and Q is a protecting group, like alkanoyl, e.g. acetyl.

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(b) covalently reacting the intermediate with an amide linkage from step (a) with an activated polyethylene glycol derivative represented by the formula, $W-[OCH_2CH_2]_m-OR$, to form an NK4 protein product represented by the formula:



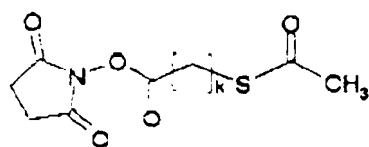
5 wherein W is a sulfhydryl reactive form of Y; m is an integer ranging from about 200 to about 950; R is lower alkyl; and Y is as defined above.

In this embodiment, the bi-functional reagent is preferably N-succinimidyl-S-acetylthiopropionate or N-succinimidyl-S-acetylthioacetate, Z is preferably N-hydroxy-succinimide, and the activated polyethylene glycol derivative $W-[OCH_2CH_2]_m-OR$ is
10 preferably selected from the group consisting of iodo-acetyl-methoxy-PEG, methoxy-PEG-vinylsulfone, and methoxy-PEG-maleimide.

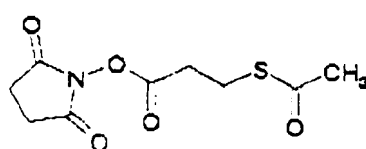
In more detail, the NK4 proteins of formula (III) may be prepared by covalent linking of thiol groups to NK4 ("activation") and coupling the resulting activated NK4 with a poly(ethylene glycol) (PEG) derivative. The first step for the preparation of pegylated NK4
15 according to the present invention comprises covalent linking of thiol groups via NH_2 -groups of NK4. This activation of NK4 is performed with bi-functional reagents which carry a protected thiol group and an additional reactive group, such as active esters (e.g., a succinimidylester), anhydrides, esters of sulphonic acids, halogenides of carboxylic acids and sulphonic acids, respectively. The thiol group is protected by groups known in the art,
20 e.g., acetyl groups. These bi-functional reagents are able to react with the ξ -amino groups of the lysine amino acids by forming an amide linkage.

In a preferred embodiment the activation of the ϵ -amino lysine groups is performed by reaction with bi-functional reagents having a succinimidyl moiety. The bi-functional reagents may carry different spacer species, e.g. $-(CH_2)_k-$ or $-CH_2-(O-CH_2-CH_2-)_k-$
25 moieties, wherein k is from 1 to about 10, preferably from 1 to about 4, and more preferably 1 or 2, and most preferably 1. Examples of these reagents are N-succinimidyl-S-acetylthiopropionate (SATP) and N-succinimidyl-S-acetylthioacetate (SATA)

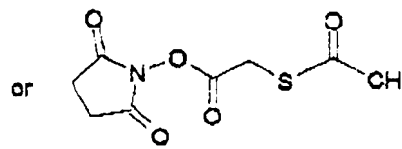
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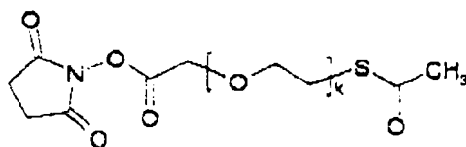
Acetylthioalkyl-carboxylic-NHS-ester, like



SATP



SATA

2-(Acetylthio)-(ethoxy)_k-acetic-acid-NHS-ester

with k as defined above.

The preparation of the bi-functional reagents is known in the art. Precursors of 2-(acetylthio)-(ethoxy)_k-acetic-acid-NHS-esters are described in DE-3924705, while the derivatization to the acetylthio compound is described by March, J., Advanced Organic Chemistry (1977) 375-376. SATA is commercially available (Molecular Probes, Eugene, OR, USA and Pierce, Rockford, IL).

The number of thiol groups to be added to an NK4 molecule can be selected by adjusting the reaction parameters, i.e., the protein (NK4) concentration and the protein/bi-functional reagent ratio. Preferably, the NK4 is activated by covalently linking from 1 to 5 thiol groups per NK4 molecule, more preferably from 1.5 to 3 thiol groups per NK4 molecule. These ranges refer to the statistical distribution of the thiol group over the NK4 protein population.

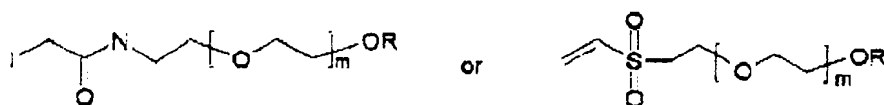
The reaction is carried out, for example, in an aqueous buffer solution, pH 6.5-8.0, e.g., in 10 mM potassium phosphate, 300 mM NaCl, pH 7.3. The bi-functional reagent may be added in DMSO. After completion of the reaction, preferably after 30 minutes, the reaction is stopped by addition of lysine. Excess bifunctional reagent may be separated by

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methods known in the art, e.g., by dialysis or column filtration. The average number of thiol groups added to NK4 can be determined by photometric methods described in, for example, Grasseti, D.R., and Murray, J.F. in *J. Appl. Biochem. Biotechnol.* 119 (1967) 41-49.

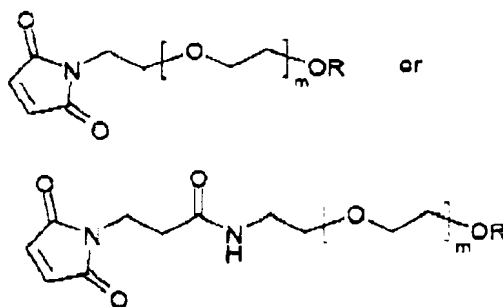
- 5 The above reaction is followed by covalent coupling of an activated polyethylene glycol (PEG) derivative. Suitable PEG derivatives are activated PEG molecules with an average molecular weight of from about 10 to about 40 kDa, more preferably from about 20 to about 40 kDa.

- Activated PEG derivatives are known in the art and are described in, for example,
 10 Morpurgo, M., et al. *J. Bioconj. Chem.* 7 (1996) 363 ff for PEG-vinylsulfone. Linear chain and branched chain PEG species are suitable for the preparation of the compounds of Formula 1. Examples of reactive PEG reagents are iodo-acetyl-methoxy-PEG and methoxy-PEG-vinylsulfone:



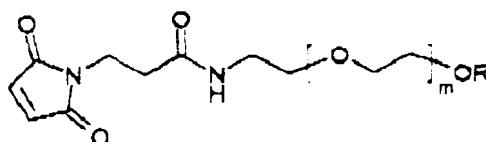
- 15 The use of these iodo-activated substances is known in the art and described e.g. by Hermanson, G.T., in *Bioconjugate Techniques*, Academic Press, San Diego (1996) p. 147-148.

- Most preferably, the PEG species are activated by maleimide using (alkoxy-PEG-maleimide), such as methoxy-PEG-maleimide (MW 10000 to 40000; Shearwater Polymers,
 20 Inc.). The structure of alkoxy-PEG-maleimide is as follows:



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with R and m are as defined above, preferably



The coupling reaction with alkoxy-PEG-maleimide takes place after *in situ* cleavage of the thiol protecting group in an aqueous buffer solution, e.g. 10 mM potassium phosphate, 300 mM NaCl, 2 mM EDTA, pH 6.2. The cleavage of the protecting group may be performed, for example, with hydroxylamine in DMSO at 25°C, pH 6.2 for about 90 minutes. For the PEG modification the molar ratio of activated NK4/alkoxy-PEG-maleimide should be from about 1:1 to about 1:6. The reaction may be stopped by addition of cysteine and reaction of the remaining thiol (-SH) groups with N-methylmaleimide or other appropriate compounds capable of forming disulfide bonds. Because of the reaction of any remaining active thiol groups with a protecting group such as N-methylmaleimide or other suitable protecting group, the NK4 proteins in the conjugates of this invention may contain such protecting groups. Generally the procedure described herein will produce a mixture of molecules having varying numbers of thiols protected by different numbers of the protecting group, depending on the number of activated thiol groups on the protein that were not conjugated to PEG-maleimide.

Whereas N-methylmaleimide forms the same type of covalent bond when used to block the remaining thiol-groups on the pegylated protein, disulfide compounds will lead in an intermolecular sulfide/disulfide exchange reaction to a disulfide bridged coupling of the blocking reagent. Preferred blocking reagents for that type of blocking reaction are oxidized glutathione (GSSG), cysteine and cystamine. Whereas with cysteine no additional net charge is introduced into the pegylated protein, the use of the blocking reagents GSSG or cystamine results in an additional negative or positive charge.

25 The further purification of the compounds of formula (III), including the separation of mono-, di- and tri-pegylated NK4 species, may be done by methods known in the art, e.g., column chromatography.

Usually mono-PEG conjugates of NK4 proteins are desirable because they tend to have higher activity than di-PEG conjugates.. The percentage of mono-PEG conjugates as well as

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the ratio of mono- and di-PEG species can be controlled by pooling broader fractions around the elution peak to decrease the percentage of mono-PEG or narrower fractions to increase the percentage of mono-PEG in the composition. About ninety percent mono-PEG conjugates is a good balance of yield and activity. Sometimes compositions in which, for example, at least ninety-two percent or at least ninety-six percent of the conjugates are mono-PEG species (n equals 1) may be desired. In an embodiment of this invention the percentage of conjugates where n is 1 is from ninety percent to ninety-six percent.

Pharmaceutical formulations

Pegylated NK4 can be administered as a mixture, or as the ion exchange chromatography or size exclusion chromatography separated different pegylated species. The compounds of the present invention can be formulated according to methods for the preparation of pharmaceutical compositions which methods are known to the person skilled in the art. For the production of such compositions, pegylated NK4 according to the invention is combined in a mixture with a pharmaceutically acceptable carrier. Such acceptable carriers are described, for example, in Remington's Pharmaceutical Sciences, 18th edition, 1990, Mack Publishing Company, edited by Oslo et al. (e.g. pp. 1435-1712). Typical compositions contain an effective amount of the substance according to the invention, for example from about 0.1 to 100 mg/ml, together with a suitable amount of a carrier. The compositions may be administered parenterally.

This invention further provides pharmaceutical compositions containing conjugates described herein in which the percentage of conjugates in which n is 1, 2 and/or 3 is preferably at least ninety percent, more preferably at least ninety-two percent.

The pharmaceutical formulations according to the invention can be prepared according to known methods in the art. Usually, solutions of pegylated NK4 are dialyzed against the buffer intended to be used in the pharmaceutical composition and the desired final protein concentration is adjusted by concentration or dilution.

Such pharmaceutical compositions may be used for administration for injection and contain an effective amount of the monopegylated NK4 together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer contents (e.g. arginine, acetate, phosphate), pH and ionic strength, additives such as detergents and solubilizing agents (e.g. Tween 80/polysorbate, pluronic F68), antioxidants (e.g. ascorbic acid, sodium metabisulfite),

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preservatives (Timersol, benzyl alcohol) and bulking substances (e.g. saccharose, mannitol), incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Such compositions may influence the physical state stability rate of release and clearance of the
5 monopegylated NK4 according to the invention.

Dosages and drug concentrations

Typically, in a standard cancer treatment regimen, patients are treated with dosages in the range between 0.01 to 3 mg of pegylated NK4 per kg per day over a certain period of time, lasting from one day to about 30 days or even longer. Drug is applied as a single daily
10 subcutaneous or i.v. bolus injection of a pharmaceutical formulation containing 0.1 to 100 mg pegylated NK4 per ml. This treatment can be combined with any standard (e.g. chemotherapeutic) treatment, by applying pegylated NK4 before, during or after the standard treatment. This results in an improved outcome compared to standard treatment alone.

15 The following examples, references and the sequence listing are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

- SEQ ID NO:1 shows the DNA and polypeptide sequence of NK4.
- 20 SEQ ID NO:2 shows the polypeptide sequence of NK4.

Example 1

Recombinant production of NK4

NK4 for therapeutic uses may be produced by recombinant means using bacterial or eukaryotic expression systems. Suitable eukaryotic expression systems are for example
25 engineered HeLa, BHK or preferably CHO cells. Cells engineered for NK4 production are cultivated in a suitable medium. Typically, a 1 to 5 liter cell culture is used as inoculum for a 10 liter fermenter. After 3 to 5 days, the culture in the 10 liter fermenter can be used as inoculum for the 100 liter fermenter. After additional 3 to 5 days of fermentation, this culture can be used as inoculum for the 1000 liter production fermenter. After 3 to 4 days
30 cells are removed by filtration or centrifugation and discarded. The NK4 containing

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supernatant is filtered, collected and processed during purification. The purification process is described in the following example.

Example 2

Purification

- 5 Heparin-Sepharose consists of Sepharose beads to the surface of which heparin is covalently bound. Since NK4 shows a high affinity to heparin it is retained on this column and can be eluted with high salt concentrations, whereas protein contaminants and other impurities either do not bind or elute at lower salt concentrations. NK4 containing fractions, eluting at about 0.7 to 1.1 M NaCl in 50 mM Hepes pH 7.5 are collected and loaded onto a
- 10 hydroxyapatite column. NK4 elutes with about 0.4 to 0.7 M potassium phosphate, pH 7.5. The resulting fractions are substantially free of contaminating proteins and can be further purified by Q-sepharose chromatography.

Example 3

Production of pegylated NK4

- 15 NK4 purified in accordance with the above mentioned procedure was used for pegylation reactions. Two of the above-mentioned suitable methods are exemplarily described.

a) Pegylation of NK4 with mPEG-SBA

- Aliquots of NK4 were reacted with methoxy-PEG-SBA (10 kDa, 20 kDa, 30 kDa and 40 kDa, respectively; Shearwater Polymers, Inc., Huntsville Alabama). Reaction was carried
- 20 out at a protein to reagent ratio between 1:1 and 1:5 for about 2 h at room temperature. The reaction was stopped by the addition of 30 mM Tris-buffer and samples were analyzed by SDS-PAGE or size exclusion chromatography on a Superose 6 column (Pharmacia) using as buffer solution 50 mmol/l Na phosphate, 300 mmol/l NaCl, pH 6.8, for equilibration and elution. The reaction was optimized by varying protein to reagent ratio,
- 25 pH, time and temperature, in order to obtain predominantly mono-pegylated NK4.

b) Pegylation of NK4 with mPEG-SPA

Aliquots of NK4 were reacted with methoxy-PEG-SPA (10 kDa, 20 kDa, 30 kDa and 40 kDa, respectively; Shearwater Polymers, Inc., Huntsville Alabama). Reaction was carried out at a protein to reagent ratio between 1:1 and 1:5 for about 2 h at room temperature.

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The reaction was stopped by the addition of 30 mM Tris-buffer and samples were analyzed by SDS-PAGE or size exclusion chromatography on a Superose 6 column (Pharmacia) using as buffer solution 50 mmol/l Na phosphate, 300 mmol/l NaCl, pH 6.8, for equilibration and elution. The reaction was optimized by varying protein to reagent ratio, pH, time and temperature, in order to obtain predominantly mono-pegylated NK4, compared to di- and tri-pegylated NK4.

Example 4

Isolation of monopegylated NK4

Monopegylated NK4 can be separated from unpegylated, di- and tri-pegylated NK4 by running a preparative size exclusion chromatography (e.g. Superose 6 or Superdex 200; Pharmacia) using as buffer solution 50 mmol/l Na phosphate, 300 mmol/l NaCl, pH 6.8, for equilibration and elution, or by ion exchange chromatography. The purified protein contains predominantly the mono-pegylated species. Fractions were collected and analyzed by SDS-PAGE.

Example 5

Molecular characterization of mono-pegylated NK4

a) Size exclusion chromatography

The mono-pegylated species elutes earlier in size exclusion chromatography (e.g. Superose 6 or Superdex 200; Pharmacia; using as buffer solution 50 mmol/l Na phosphate, 300 mmol/l NaCl, pH 6.8, for equilibration and elution) as compared to the unmodified form. This is due to an increased hydrodynamic radius of the molecule.

b) SDS-PAGE

In SDS-PAGE proteins are separated according to their molecular weight. Due to an increase in molecular weight by pegylation, the mono-pegylated NK4 shows a shorter migration distance as compared to the unmodified NK4. The migration distance is inversely correlated with the chain length of the PEG moiety and the number of PEG groups attached per NK4 molecule.

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c) **Peptide mapping**

Digestion of pegylated NK4 with sequence-specific endo-proteinases (e.g. LysC or trypsin) results in a characteristic peptide map. The resulting peptides can be separated by reversed phase chromatography and analyzed by mass spectrometry and/or N-terminal sequencing.

5 This allows for a determination of the PEG-modified groups within the NK4 molecule.

d) **Reverse phase chromatography**

Pegylated NK4 can also be characterized by reversed phase chromatography. Pegylation of NK4 results in a change in retention time as compared to unmodified NK4.

Example 6

10 **Comparison of monopegylated, unpegylated and multi-pegylated NK4**

a) **Scatter assay**

MDCK cells were subconfluently grown in tissue culture plates. Cells were treated with HGF (10 ng/ml) or with combinations of HGF and NK4 (un-, mono-, or multi-pegylated). In these experiments the HGF-induced cell scattering was inhibited by the addition of a 10
15 to 1000-fold molar excess of NK4 (un-, mono-, or multi-pegylated), showing the functional activity of pegylated NK4.

b) **Proliferation assay**

Inhibition of the mitogenic activity of HGF by NK4 (un-, mono-, or multi-pegylated) was determined by measuring DNA synthesis of adult rat hepatocytes in primary culture as
20 described in Nakamura et al. (1989). In these experiments the HGF-induced cell proliferation was inhibited by the addition of a 10 to 1000-fold molar excess of NK4 (un-, mono-, or multi-pegylated), showing the functional activity of pegylated NK4.

c) **Invasion assay**

In this assay the invasive potential of tumor cells is analyzed. The assay was done essentially
25 as described in Albini et al. (1987) using HT115 cells. Again, HGF-induced (10 ng/ml) cell invasion could be inhibited by a 10 to 1000-fold molar excess of NK4 (un-, mono-, or multi-pegylated), showing the functional activity of pegylated NK4.

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Example 7

Activity in vivo

Model: Lewis Lung Carcinoma nude mouse tumor model

5 1×10^6 lewis lung carcinoma cells were s.c. implanted into male nude mice (BALB/c nu/nu).

Treatment: After 4 days, one application daily of pegylated NK4 over a period of 2-4 weeks

Dose: 1000 $\mu\text{g}/\text{mouse}/\text{day}$

300 $\mu\text{g}/\text{mouse}/\text{day}$

100 $\mu\text{g}/\text{mouse}/\text{day}$

10 placebo

Result: Treatment with pegylated NK4 shows a dose dependent suppression of primary tumor growth and metastasis, whereas no effect is seen in placebo treated groups.

15 Example 8

Pharmaceutical composition

Suitable pharmaceutical compositions are, for example:

1 to 30 mg/ml pegylated NK4

150 mM NaCl

20 10 mM sodium phosphate, pH 7.2

1 to 30 mg/ml pegylated NK4

150 mM NaCl

0.01% Tween 80 or Tween 20 or pluronic F68

25 10 mM sodium phosphate, pH 7.2

1 to 30 mg/ml pegylated NK4

50 mM NaCl

3% mannitol

30 10 mM sodium phosphate, pH 7.2

1 to 30 mg/ml pegylated NK4

50 mM NaCl

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3% mannitol

0.01% Tween 80 or Tween 20 or pluronic F68

10 mM sodium phosphate, pH 7.2

- 5 The compositions are prepared in that pegylated NK4 is dialyzed against the above mentioned buffer solution (with or without mannitol). The protein concentration is adjusted by concentration or dilution with the buffer solution. Detergent is added out of a 10% stock solution.

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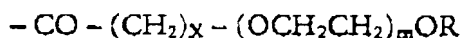
List of References

- Albini et al., Cancer Res. 47 (1987) 3239-3245
- Chamow et al., Bioconjugate Chem. 5 (1994) 133-140
- Date, K., et al., FEBS Letters 420 (1997) 1-6
- 5 Date, K., et al., Oncogene 17 (1989) 3045-3054
- DE 3924705
- Delgano et al., in: Coupling of PEG to protein by activation with tresylchloride, applications in immunoaffinity cell preparation (eds. Fischer et al.), Separations using aqueous phase systems, applications in cell biology and biotechnology, Plenum Press, New York, 1989, pp. 211-213
- 10 EP 0 154 316
- EP 0 401 384
- EP 0 442 724
- EP 0 539 167
- 15 EP 0 822 199
- Francis et al. in Stability of Protein Pharmaceuticals: in vivo Pathways of Degradation And Strategies for Protein Stabilization (eds. Ahern, T., and Manning, M.C.), Plenum Press, New York, 1991
- Gaertner, H.F., and Offord, R.E., Bioconjugate Chem. 7 (1996) 38-44
- 20 Grasetti, D.R., and Murray, J.F., J. Appl. Biochem. Biotechnol. 119 (1967) 41-49
- Hermanson, G.T., et al., Bioconjugate Techniques, Academic Press, San Diego, (1996) 147-148
- Kuba, K., et al., Cancer Res. 60 (2000) 6737-6743
- March, J., Advanced Organic Chemistry (1977) 375-376
- 25 Mehvar, R., J. Pharm. Pharm. Sci. 3 (1) (2000) 125-136
- Miyazawa, K., et al., Biochem. Biophys. Res. Comm. 163 (1989) 967-973
- Morpurgo, M., et al., Bioconj. Chem. 7 (1996) 363
- Nakamura, T., et al., Biochem. Biophys. Res. Commun. 22 (1984) 1450-1459
- Nakamura, T., et al., Nature 342 (1989) 440-443
- 30 Okajima, A., et al., Eur. J. Biochem. 193 (1990) 375-381
- Parr, C., et al., Int. J. Cancer 85 (2000) 563-570
- Remington's Pharmaceutical Sciences, 18th edition, 1990, Mack Publishing Company, edited by Oslo et al. (e.g. pp. 1435-1712)
- Seki, T., et al., Biochem. and Biophys. Res. Comm. 172 (1990) 321-327
- 35 Stuart, K.A., et al., International Journal of Experimental Pathology 81 (2000) 17-30
- Tashiro, K., et al., Proc. Natl. Acad. Sci. USA 87 (1990) 3200-3204

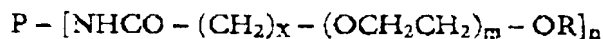
- 23 -

- U.S. Patent No. 4,002,531
- U.S. Patent No. 4,904,584
- U.S. Patent No. 5,252,714
- U.S. Patent No. 5,382,657
- 5 U.S. Patent No. 5,672,662
- U.S. Patent No. 5,977,310
- Weidner, K.M., et al., Proc. Natl. Acad. Sci. USA 88 (1991) 7001-7005
- WO 01/02017
- WO 90/04606
- 10 WO 90/07938
- WO 93/23541
- WO 94/01451

5. A conjugate according to claim 1, characterized in that said polyethylene glycol group(s) has/have the formula



- 20 6. A conjugate according to claim 5 having the formula I



wherein

X is 2 or 3;

m is from about 200 to about 950;

25 n is 2 or 3;

P is said N-terminal fragment of hepatocyte growth factor without the n amino group(s) which form amide linkage(s) with the poly(ethylene glycol) group(s).

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7. A pharmaceutical composition comprising a conjugate of claims 1 to 6 and a pharmaceutically acceptable carrier.
8. A process for preparing a pharmaceutical composition according to claim 7.
9. Use of a conjugate according to claims 1 to 6 for the preparation of a medicament useful in the treatment of cancer.
- 5 10. Method for the treatment of cancer diseases, comprising the steps of administering to a patient in need thereof a pharmaceutical composition according to claim 7.

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Abstract

A conjugate comprising an N-terminal fragment of hepatocyte growth factor (HGF/SF) consisting of the hairpin domain and the four kringle regions of the α -chain and one to three polyethylene glycol group(s), said polyethylene glycol group(s) having an overall molecular weight of from about 10 to 40 kDa, has improved properties and is a useful therapeutic agent for tumor treatment.

- 1 -

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	Thr	Asp	Pro	Asn	Ile	Arg	Val	Gly	Tyr	Cys	Ser	Gln	Ile	Pro	Asn	Cys
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	Asp	Met	Ser	His	Gly	Gln	Asp	Cys	Tyr	Arg	Gly	Asn	Gly	Lys	Asn	Tyr
		355						360					365			
35	Met	Gly	Asn	Leu	Ser	Gln	Thr	Arg	Ser	Gly	Leu	Thr	Cys	Ser	Met	Trp
	370						375					380				
	Asp	Lys	Asn	Met	Glu	Asp	Leu	His	Arg	His	Ile	Phe	Trp	Glu	Pro	Asp
	385					390					395					400
40	Ala	Ser	Lys	Leu	Asn	Glu	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Asp	Asp	Ala
				405						410					415	
	His	Gly	Pro	Trp	Cys	Tyr	Thr	Gly	Asn	Pro	Leu	Ile	Pro	Trp	Asp	Tyr
			420					425						430		
	Cys	Pro	Ile	Ser	Arg	Cys	Glu	Gly	Asp	Thr	Thr	Pro	Thr	Ile	Val	
			435					440						445		
45																